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Sex-independent suppression of experimental inflammatory pain by minocycline in two mouse strains

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HIGHLIGHTS

- Minocycline suppresses inflammatory pain irrespective of mouse sex and strain.
- Lack of motor coordination does not account for suppression of nociceptive response.
- These results may be translated to minocycline's analgesic actions in men and women.

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ABSTRACT

The research on sex differences in nociception and antinociception as well as sex and gender differences in pain and analgesia is a maturing field. There is a vast literature showing experimental and clinical pain suppressive effects induced by minocycline, especially in inflammatory pain. However, as far as we know, possible qualitative or quantitative sex differences in those effects remained to be examined. By employing the formalin test, which has two phases of experimental pain behavior that models nociceptive pain (i.e., first phase) and inflammatory pain (i.e., second phase), we initially evaluated the effect induced by minocycline in female or male C57BL/6 mice. The treatment reduced the second phase of licking behavior in both females and males, and the effects were quantitatively similar in both sexes. Likewise, the same sex-independent effect was observed in Swiss mice, suggesting a genotype-unspecific sex-independent effect. While minocycline is already being tested in clinical trials, this appears to be the first preclinical investigation of sex differences in the experimental pain suppressive effects induced by this widely studied drug. The independence of sex in the antinociceptive effect induced by minocycline may be hopefully translated to gender-independent analgesic effects, which would be surely promising in a therapeutic paradigm.

1. Introduction

Data from many studies show that pain/nociception and analgesia/antinociception, both in humans and experimental animals, exhibit great interindividual variability and the factors that may contribute to such differences have been intensely investigated recently [18]. Furthermore, data from large epidemiological studies clearly show that pain is more prevalent in women than in men [21]. Nevertheless, there is a clear male-orientated bias in experimental subject choice in the field of pain and others; and the reasons for this bias are believed to be the inertia of pain researchers and their over-concern about estrous cycle-related variability [18]. The inclusion of females is mandatory in clinical but not in preclinical studies. For this reason, the awareness of this matter has been increasing and the investigation of sex and gender differences in nociception, pain and inflammation and their inhibition has been maturing [6,12]. Currently and more frequently, sex is used to refer to a biologically based dichotomous variable, whereas gender refers to a socially based phenomenon, considered to range from exclusively feminine to exclusively masculine. Therefore, in human studies, group differences are likely to be attributable to either sex or gender. On the other hand, only the term "sex" applies to preclinical data and will be used throughout this manuscript [12].

Clinical trials have been conducted to test the clinical utility of repositioning minocycline as an analgesic or antirheumatic drug [11,20,30]. Indeed, this second-generation tetracycline exhibits anti-inflammatory effects unrelated to its antibacterial one [1], and anti-inflammatory effects help explain either its antinociceptive [2]

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or neuroprotective actions [16,27]. Minocycline is neuroprotective after experimental stroke, a pathological condition characterized by massive inflammatory response [29]. A study suggested that the neuroprotective effect in the middle cerebral artery occlusion model is dependent on sex in C57BL/6 mice [19], whereas another study suggested that neuroprotective effects in a thromboembolic stroke model are independent of sex in the same mouse strain [13]. Therefore, we wondered whether there might be qualitative or quantitative sex differences in the effect induced by minocycline on inflammatory pain.

There is a very high number of studies showing suppression of experimental pain by tetracycline derivatives in rodents, especially inflammatory pain [2]. However, as far as we know, just in a few studies the authors clearly informed the use of female animals only [3,24,32], and therefore a "head-to-head" comparison between the effects induced by minocycline in females and males remained to be performed. Then, we designed a straightforward study to compare the effect induced by minocycline in female and male mice using the formalin test, which models both nociceptive (i.e., first phase of licking behavior) and inflammatory (i.e., the second phase) pain [14,31]. To evaluate whether a sex difference is a genotype specific or unspecific effect, we evaluated the activity of minocycline in two mouse strains.

2. Materials and methods

2.1. Animals

Seven to nine week-old C57BL/6 or Swiss mice of both sexes were used. Efforts were made to minimize both animal distress and the number of animals used. The animals had free access to food and water and were maintained in a room with a 12 h light–dark cycle. The experiments were performed at room with temperature controlled between 26 and 28 °C, which ranges within the thermoneutral zone for mice [10] and it is suitable for carrying out the temperature-sensitive formalin test [31]. All experiments were performed according to the ethical guidelines for the investigation of experimental pain in conscious animals [33]. Experimenters were blinded to treatments. Each experiment was performed in a separate group of mice.

2.2. Drugs

Minocycline hydrochloride (Galena, Campinas, Brazil), formaldehyde 37% (m/v) (Sigma–Aldrich, St Louis, MO, USA) and phenobarbital (Aventis Pharma, São Paulo, Brazil) were used. Solutions and suspensions were prepared in isotonic saline immediately before the intraperitoneal (i.p.) injections. The volume injected was 5 ml/kg.

2.3. Effect induced by minocycline on the nociceptive response induced by formalin in mice

For three consecutive days before the experiment, the animals were habituated for approximately 30 min to the testing apparatuses to minimize stress-induced antinociception on the testing day. Each mouse was placed under a transparent glass funnel (18 cm diameter, 15 cm high). Formalin (2.5%, v/v, 20 μl) was injected subcutaneously (s.c.) into the dorsum of the right hind paw of mice 1 h after the i.p. administration of minocycline (12.5, 25, 50 or 100 mg/kg) or saline. This minocycline dose range was chosen on the basis of previous studies performed by our research group and others [1,3–5,25]. The amount of time the animal intermittently licked the injected paw was counted manually by using stopwatches between 0 and 5 min (first phase) and 15 and 30 min (second phase) after the injection of formalin.

2.4. Evaluation of the motor coordination of mice in the rota-rod

The motor coordination of the animals was evaluated in a rota-rod apparatus. This test is essential when studying experimental pain to examine the possibility of reduced display of nociceptive behavior due to lack of motor coordination that may result from central nervous system depression or muscle relaxation [9,15].

The animals were trained on the apparatus for three days before the experiment. On the testing day, the animals were placed on the rotating rod (12 r.p.m.) and the latency to fall was measured. The cut-off time was 120 s. After confirming that all animals were sufficiently trained to stay on the rotating rod for at least 120 s, they were treated with minocycline (100 mg/kg, i.p.) or phenobarbital (40 mg/kg; i.p., positive control) and 1 h later they were again tested on the apparatus. Phenobarbital, a central nervous system depressant, was used as a positive control because of its well-known ability to impair the performance of rodents in the rotarod test [1,4,7].

2.5. Data analysis

The pain behavior data were analyzed by two-way ANOVA, taking sex and drug treatment as main factors, followed by Bonferroni post hoc test for multiple comparisons. Calculations of half-maximal inhibitory doses (ID_{50}) were performed by using the software JFlashCalc (M.H. Ossipov, University of Arizona, AZ, USA). As rota-rod data did not display normal distribution (analyzed by Kolmogorov–Smirnov test), Kruskal–Wallis test, followed by Dunn's multiple comparison test, was applied. Values of $p < 0.05$ were considered to show significant differences between means or medians. The software Prism® 5 (GraphPad Software Inc., San Diego, CA, USA) was used for such analyses. For ease of reading, the basic statistical values are shown in the text, whereas the more extensive statistical information can be found in the figure captions.

3. Results

The s.c. injection of formalin (2.5%, 20 μl) in mice induced a biphasic nociceptive response characterized mainly by licking the injected paw. Fig. 1A and 1B show that the first phase of licking behavior was unaffected by minocycline treatment in C57BL/6 mice, whereas the second phase was attenuated by this tetracycline derivative, as revealed by two-way ANOVA. However, the minocycline's antinociceptive activity was not affected by sex. Reinforcing the sex-independent effect induced by minocycline in this experimental setting, minocycline inhibited the second phase of licking behavior in either female or age-matched male mice of a different mouse strain (i.e., Swiss mice; Fig. 2B), whereas the first phase was unaffected (Fig. 2A). In male C57BL/6 and female Swiss mice, the half-maximal inhibitory doses (ID_{50}) were 89 mg/kg (95% confidence interval: 20–386 mg/kg) and 61 mg/kg (95% confidence interval: 24–155 mg/kg), respectively. By extrapolation, the ID_{50} values in female C57BL/6 and male Swiss mice were 123 and 140 mg/kg, respectively; however, these estimated numbers should be treated cautiously because the highest dose used in the present study was 100 mg/kg (i.p.). We have not tried higher doses because of the risk of toxicity – 100 mg/kg is already a high dose. Indeed, as far we know, there is no study on experimental pain that used a dose of minocycline higher than 100 mg/kg (i.p.).

Importantly, lack of motor coordination seems not to account for reduced display of experimental pain behavior, as the highest dose of minocycline (100 mg/kg) did not affect the performance of mice in the rota-rod test (Table 1). On the other hand, phenobarbital, used

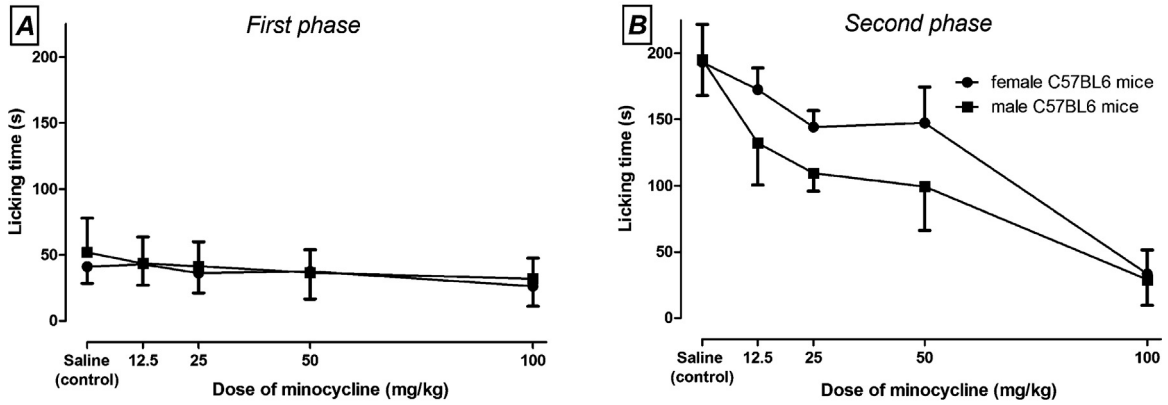

Fig. 1. Effects induced by minocycline (12.5, 25, 50 or 100 mg/kg or saline, i.p., −1 h) on the first (A) and second (B) phase of nociceptive response induced by formalin in female or male C57BL/6 mice. (A) No effect of treatment on both sexes in the first phase ($p > 0.05$). (B) Main effect of treatment, $F_{(4,73)} = 13.40$, $p < 0.0001$; main effect of sex, $F_{(1,73)} = 2.78$, $p = 0.1000$; interaction, $F_{(4,73)} = 0.45$, $p = 0.7707$. Data are expressed as mean ± SEM ($n = 8$–9), analyzed by two-way ANOVA followed by Bonferroni post hoc test, taking treatment and sex as main factors. The Bonferroni test reveals no significant statistical difference between females and males at any dose (all p values higher than 0.05).

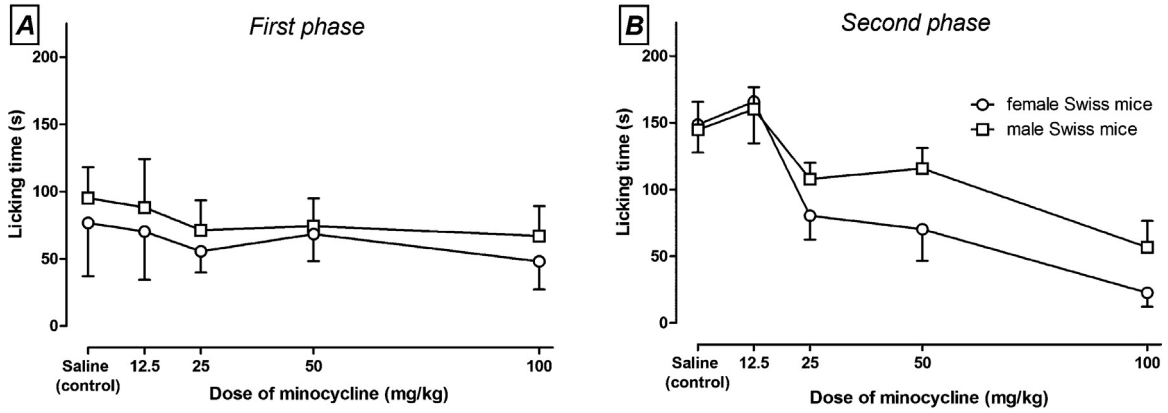

Fig. 2. Effects induced by minocycline (12.5, 25, 50 or 100 mg/kg or saline, i.p., −1 h) on the first (A) and second (B) phase of nociceptive response induced by formalin in female or male Swiss mice. (A) No effect of treatment on both sexes ($p > 0.05$). (B) Main effect of treatment, $F_{(4,71)} = 12.28$, $p = 0.0001$; main effect of sex, $F_{(1,71)} = 2.44$, $p = 0.1227$; interaction, $F_{(4,71)} = 0.68$, $p = 0.6059$. Data are expressed as mean ± SEM ($n = 8$–9), analyzed by two-way ANOVA followed by Bonferroni test, taking treatment and sex as main factors. The Bonferroni test reveals no significant statistical difference between females and males at any dose (all p values higher than 0.05).

as a positive control, inhibited the performance in comparison with vehicle- or minocycline-treated animals.

4. Discussion

Although the experimental pain suppressive effects induced by minocycline have been widely studied [2], this seems to be the first study to examine possible sex differences in this context. Minocycline's beneficial actions on experimental inflammatory and neuropathic pain have been shown, but this drug seems not to suppress nociceptive pain. In the present study, we show that inflammatory pain is suppressed by minocycline in a sex-independent manner in C57BL/6, the most widely used mouse strain. We used a second mouse strain (i.e., Swiss) and observed similar results, thus reinforcing that the sex-independent effect induced by minocycline is not restricted to a single mouse strain. Indeed, the genotype makes difference when studying sex differences in pain. Because of this, some investigators fail to observe sex differences that others document clearly, using robust statistical analysis [22].

Table 1
Effects induced by saline (vehicle), minocycline or phenobarbital (positive control) on the motor coordination of female or male C57BL/6 or Swiss mice.

Strain and sex		Latency (s) to fall 1 h after the following treatments (i.p.)								
		Saline			Mino 100 mg/kg			Pheno 40 mg/kg		
		25%	Median	75%	25%	Median	75%	25%	Median	75%
C57BL/6	♀	111.0	120	120	64.5	120	120	6	19.5[a,b]	30.75
C57BL/6	♂	120	120	120	120	120	120	1	3[a,b]	35
Swiss	♀	120	120	120	120	120	120	3	5[a,b]	120
Swiss	♂	120	120	120	120	120	120	16	20[a,b]	120

Data are expressed as median and 25% and 75% percentiles.
Cut-off time and minimal baseline latencies to fall: 120 s
[a] Significantly different from respective saline-treated group medians ($p < 0.05$), as analyzed by Kruskal–Wallis followed by Dunn's multiple comparison tests. $n = 7$–9.
[b] Significantly different from respective minocycline-treated group medians ($p < 0.05$), as analyzed by Kruskal–Wallis followed by Dunn's multiple comparison tests. $n = 7$–9.

It is unlikely that central depression or muscle relaxation contributed to the reduced nociceptive behavior displayed by the animals treated with minocycline, as this drug did not impair their performance in the rota-rod test, irrespective of mouse sex or strain. Similar results have also been observed for minocycline, doxycycline [4] and 12*S*-hydroxy-1,12-pyrazolinominocycline (a non-antibacterial minocycline derivative) [1] in previous studies. Using the rota-rod test in a quite different experimental setting, it was observed that minocycline reverses the impaired performance of rats subjected to traumatic brain injury [28]. Although the rota-rod test is not very widely used in studies on experimental pain, its use should be more strongly encouraged because it has proved to be helpful in research involving screening of drugs that may affect motor coordination [9,15].

Sex differences in minocycline's actions have been examined in stroke models, a pathological condition with a strong inflammatory component [29], and somewhat contrasting results have been shown. It was initially suggested that the neuroprotective effect in the middle cerebral artery occlusion model is dependent on sex in C57BL/6 mice [19]. A more recent study suggested that this effect induced by minocycline on thromboembolic stroke is independent of sex in the same mouse strain [13]. Different stroke models were used in these studies, which may account for the observation of sex difference in one study but not in the other. However, in order to assess possible qualitative or quantitative sex differences related to a certain drug treatment, dose-response curves should have been built up in those studies instead of using a single dose, and the curves for females and males statistically compared with each other. Therefore, this drawback might have led to misinterpretation of sex differences in minocycline's actions on experimental stroke, and this misleading data may be counterproductive for translation from bench to bedside. Thus, appropriate pharmacological approaches and statistical analysis should be used rationally in such studies on sex differences to provide more reliable and conclusive preclinical data.

The investigation of the influence exerted by sex on nociception and antinociception in experimental animals is challenging as many factors may also be involved and contribute to erroneous interpretations. Many studies have shown that the animal strain clearly contributes to differences in the magnitude of experimental pain [17] and also the experimental pain suppressive effects induced by many drugs [23]. Moreover, other factors, that have been much less investigated, may also affect the animal's response. Among them, season and time of the day affect the response of animals to a noxious stimulus, thus providing support to the importance of chronobiological factors in the modulation of experimental pain [8,26].

Regarding the influence of sex on experimental pain, most of the investigators aim to examine the neurochemical basis of the differences found in male and female rodents, particularly the influence exerted by sex hormones [12], thus contributing to a better understanding of pathophysiology of pain. However, when it comes to the investigation of the effects induced by drugs (both experimental and those at repositioning stage) on experimental pain, there is a paucity of studies in which the authors examined their effects on both sexes. Therefore, one can easily realize that the observation of similar effects induced by a drug on both male and female experimental animals is a positive aspect that may contribute to translation and pave the road to drug development or repositioning.

As far as we know, the present study is the first preclinical study to examine sex differences in the experimental pain suppressive effects induced by a tetracycline derivative, and more studies are necessary for further elucidation of this matter. However, the demonstration of similar antinociceptive effects induced by minocycline in both sexes of two mouse strains is a positive result that may be hopefully translated to gender-independent analgesic effects, which would be promising in a clinical therapeutic paradigm.

Conflict of interest statement

The authors state no actual or potential conflict of interest whatsoever.

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